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Award Number:
    W81XWH-12-1-0479

TITLE:

Roles of microRNA-Mediated Drug Resistance in Tumor Stem Cells of Small Cell Lung Carcinoma

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REPORT DATE:

    October 2014

TYPE OF REPORT:

    Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
               Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

    x Approved for public release; distribution unlimited

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9REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)* October 2014	2. REPORT TYPE Final	3. DATES COVERED *(From - To)* 15Sep2012-14Sept2014
4. TITLE AND SUBTITLE Roles of microRNA-Mediated Drug Resistance in Tumor Stem Cells of Small Cell Lung Carcinoma		**5a. CONTRACT NUMBER** **5b. GRANT NUMBER** W81XWH-12-1-0479 **5c. PROGRAM ELEMENT NUMBER**
6. AUTHOR(S) Kounosuke Watabe, Ph.D. email: kwatabe@umc.edu		**5d. PROJECT NUMBER** **5e. TASK NUMBER** **5f. WORK UNIT NUMBER**
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Mississippi Medical Center Jackson MS 39216		**8. PERFORMING ORGANIZATION REPORT NUMBER**
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		**10. SPONSOR/MONITOR'S ACRONYM(S)** **11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Lung cancer is the leading cause of cancer deaths in the world, and according to the statistics of National Cancer Institute's SEER, 219,000 men and women will be diagnosed and 159,000 patients will die from this disease in 2014. Small cell lung carcinoma (SCLC) is the cause of approximately 20% of lung cancer. Chemo-therapy is the first choice of treatment for this carcinoma because SCLC is often highly malignant and metastasizes to the distant organs even at an early stage. Although SCLC is chemotherapy-sensitive at the initial stage, it becomes ultimately chemo-resistant with worse prognosis. Recent stem cell theory suggests that there is a distinct population of tumor cells that has stem-cell like characteristics as well as ability of resistance to chemo-therapy. Moreover, several lines of evidence indicated that the self renewal ability of cancer stem cells is regulated by microRNAs. Therefore, **we hypothesize that drug resistance of SCLC is mediated by microRNA in tumor stem cell**. In this project, we planned to identify the specific microRNA in the stem cells from SCLC. We have established a method to isolate cancer stem cells from human SCLC cell line, and our results indicate that miR16 and 21 are strongly expressed in these CSCs. We also identified five microRNAs (including miR16 and 21) that are involved in Cisplatin resistance by microRNA library screening. Our results strongly suggest that miR16 and miR21 play critical roles in chemo-resistance of cancer stem cell derived from SCLC. Therefore, these oncomirs may serve as potential biomarkers and therapeutic targets for chemoresistant SCLC.

15. SUBJECT TERMS
Small cell lung carcinoma, stem cell, resistance, microRNA

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT Unclassified	**b. ABSTRACT** Unclassified	**c. THIS PAGE** Unclassified		7	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
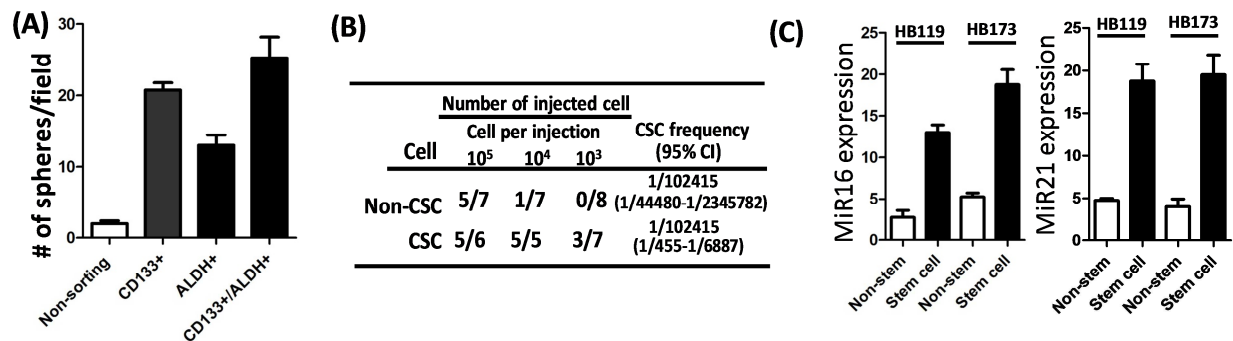Prescribed by ANSI Std. Z39.18

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INTRODUCTION

There is no cancer chemo-therapy that is 100% effective against malignant and metastatic tumors because of appearance of recurrent tumor with drug resistance. Resistance to the treatment of anti-cancer drugs is mainly attributed to the heterogeneity of cancer cells. Small cell lung carcinoma (SCLC) is one of the most malignant types of lung cancers, and has median survival of only 2-4 months from diagnosis if patients are not treated (1). This carcinoma is more responsive to chemotherapies compared to the other types of pulmonary carcinomas; however, SCLC readily acquires resistance to the drug treatment. The recent tumor stem cell theory offers an attractive explanation not only for the origin of tumors but also for the chemo-resistance mechanism (2). Moreover, several lines of evidences suggest that drug resistance and self-renewal ability of cancer stem cells are partly controlled by microRNAs (3). Therefore, it is plausible that drug resistance of SCLC is attributed to specific microRNAs in cancer stem cells. We hypothesize that drug resistance of SCLF is mediated by a specific microRNA in tumor stem cells. The purpose of this project is to identify the specific microRNA in tumor stem cells that are responsible for the drug resistance of SCLC.

BODY

Aim 1. To identify specific microRNAs in tumor stem cell from SCLC.

Progress

For this purpose, we first used two SCLC cell lines, namely HTB119 and HTB173. HTB119 was isolated from primary tumor, while HTB173 was isolated from bone metastatic site. They were cultured in RPI1640 medium, and the cells were harvested for stem cell isolation. We used a combination of CD 133 and ALDH or each marker alone for sorting cancer stem cells and found that the $CD133^+$ population or the combination of CD133 and ALDH showed significantly higher tumor imitating ability compared to

(B)

Cell	Number of injected cell			CSC frequency (95% CI)
	Cell per injection			
	10^5	10^4	10^3	
Non-CSC	5/7	1/7	0/8	1/102415 (1/44480-1/2345782)
CSC	5/6	5/5	3/7	1/102415 (1/455-1/6887)

Fig. 1. Isolation of CSCs from SCLC. (**A**) CSCs population of HTB119 and HTB173 were isolated using CD133 and ALDH as markers. They were cultured in sphere medium for 6 days and the number of spheres/field was counted. (**B**) Various numbers of CSCs were transplanted subcutaneously into the flank of nude mice and tumor incidence was counted after 30 days. (**C**) The expression of miR16 and 21 was measured in CSCs and non-stem cells in both HB119 and HB173 cells.

the ALDH-sorted cells. The final yield of ALDH+/CD133+ was approximately 3%. We tested the sphere forming ability of these cells in sphere medium in ultra-low binding plate. We found that SLDH+/CD133+ cells were capable of generating larger and more number of spheres in serum-free culture compared to the ALDH+ cells as shown in Fig. 1a. To test the tumor initiating ability of these cells, we transplanted the cells into nude mice to perform the limiting dilution analysis, and the mice were sacrificed after 30 days. We found that CD133+/ALDH+ cells of HTB173 had significantly higher ability of generating tumor in the animals (Fig. 1b).

We then extracted RNAs from these cells and they were subjected to the Affymetrix microRNA array analysis. The results of our analysis indicate that miR16 and 21 were the most significantly upregulated in stem cells of HTB173 (cut off >2.5 fold, P<0.05). We have confirmed these results by TaqMan qRT-PCR using the original stem cell populations in two cell lines (Fig. 1c). The result of a Targetscan-based analysis indicates that miR16 is capable of blocking BCL2 (anti-apoptotic gene), while miR21 targets PTEN and Jag1 that play important roles in stem cell physiology.

For the second subaim, we planned to prepare CSCs from SCLC in patients. The sample of SCLC is extremely scarce, but we managed to obtain one sample from Conversant Biologic Co. We then prepared single cell suspension followed by selecting CD133+/ALDH+ cells. We then tried to amplify these cells in NOD/SCID mice by injecting them into lungs. Unfortunately, tumor did not grow even after 2 months. Because of the time restrain, we abandoned this approach and decided to use SCLC cell lines as an alternative. We have cultured HTB119 and HTB173, and they were infected with the lentiverus-base microRNA expression library (System Biosciences). These cells were then selected for GFP$^+$ by FACS sorting to establish cell lines. We then isolated the stem cell population from these cell lines by sorting with the CD133 or ALDH marker. The sorted cells were then examined for sphere formatting abilities. CSCs (10^4 cells) were then directly transplanted into the lungs of NOD/SCID mice and let the tumor grew for two weeks followed by treating the animal with Cisplatin (3mg/kg) by intraperitoneal injection for 3 times in every 3 days. After 4 weeks, mice were sacrificed and the tumour tissue in the lungs were isolated and cell suspension was cultured in a dish. RNA was prepared from these cells and RT-PCR analysis was performed using specific primers to amplify microRNAs. At the same time, c-DNAs were subjected to sequencing analysis by Miseq (Illumina) in our core facility. The result of the sequencing analysis indicate that miR15a, miR16-1, miR21, miR155 and miR569 were all strongly amplified compared to the original tumour cells, indicating that these microRNAs are possibly involved in drug resistance of SCLC. Therefore, we accomplished Aim 1.

Aim 2. To verify the function of the identified microRNAs in tumor stem cells in vitro and *in vivo*.

Progress
We established cell lines of HTB173 that express miR15a, miR16-1, miR21, miR155 or miR569 by infecting lentivirus for these microRNA (Open Bioscience) followed by puromycine selection. The CTC population (CD133+/ALDH+) was prepared from these cells, and they were seeded in a 24-well plate followed by treating the culture with Cisplatin (20 µM) for 3 days. They were then collected, washed and re-seeded in a 96-well plate. After 24hrs, these cells were further cultured in the medium containing 20 µM of Cisplatin, and MTS assay was performed at Day 3,6 and 9. As shown in Fig. 2, we found that miR16-1, miR21 expression in these cells exhibited significant resistance to Cisplatin. Therefore, our results indicate that these microRNAs are involved in Cisplatin resistance and recurrence of SCLC.
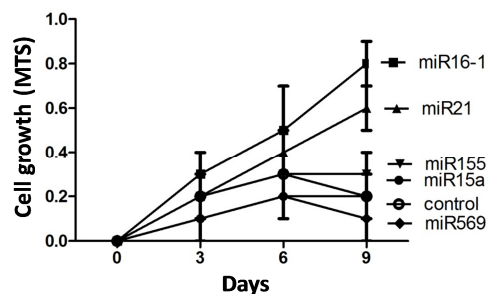
The next step is to test this hypothesis in an animal model by injecting these cells into nude mice followed by treating them with Cisplatin. This experiment is currently underway.

Fig. 2. Mir16 and 21 confers drug resistance of SCLC. The CTC population (CD133$^+$/ALDH$^+$) was prepared from HTB173 that expresses miR15a, miR16-1, miR21, miR155 or miR569. They were seeded in a 24-well plate followed by treating the cells with Cisplatin (20 μM) for 3 days. They were then collected, washed and re-seeded in a 96-well plate. After 24hrs, these cells were further cultured in the medium containing Cisplatin (20 μM), and the MTS assay was performed at Day 3 ,6 and 9.

KEY RESEARCH ACCOMPLISHMENTS

1. We have successfully isolated CSCs from small cell lung carcinoma and found that they have strong tumor-initiating capability.
2. The result of global expression analysis of these stem cells revealed that miR16 and 21 are significantly up-regulated in the CSCs of SCLC.
3. The results of microRNA library screening revealed that miR15a, miR16-1, miR21, miR155 and miR569 are significantly correlated to Cisplatin resistance of SCLC.

REPORTABLE OUTCOMES

Peer reviewed publications:
None

Employment
1. Kerui Wu (Graduate student) has been partly supported by the current grant.

CONCLUSIONS
We have established a method of isolating cancer stem cells from human SCLC. They are highly tumorigenic in animal and generate sphere formation *in vitro*. The highly metastatic stem cells express significantly higher amount of miR16 and miR21 that have been characterized as oncomirs. We also screened a microRNA library and found that miR15a, miR16-1, miR21, miR155 and miR569 were correlated to the Cisplatin resistance. Ectopic expression of miR16 and 21 indeed significantly promoted drug resistance of SCLC *in vitro*.

So what?
Although combined modality therapies have shown significant improvement of long-term survival of SCLC patients, the overall survival rate of this cancer is still much lower than other types of lung carcinoma mainly due to the recurrent tumor with drug resistance. Our results indicate that miR16 and miR21 play critical roles in chemo-resistance of cancer stem cells of SCLC. Therefore, these microRNAs may serve as potential biomarkers and therapeutic targets for SCLC. We believe that the results of this project which is uniquely focused on mircoRNAs in tumor stem cells of chemo-resistant SCLC shed new light into the drug-resistance mechanism and opened a possibility of developing a novel therapeutic drug for a better treatment of SCLC.

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